Supplement 2

Analysis of DSS samples for designer stimulants and opioids.

Chemicals and reagents

Propan-2-ol (Rotisolv®, ≥ 99.95%, LC-MS grade) and formic acid (HCOOH, > 98%, p.a.) were obtained from Carl Roth (Karlsruhe, Germany), while acetonitrile (ACN, HPLC-Super gradient grade) was purchased from VWR International (Fontenay-sous-Bois, France). Ammonium formate (10 M, 99,995%) and hydrochloric acid (HCl; ≥ 37%, p.a.) were obtained from Sigma Aldrich (Steinheim, Germany). Methanol (MeOH, Chromasolv™, LC-MS, ≥ 99.9%) was purchased from Honeywell Riedel-de Haën (Seelze, Germany), while deionized water (H_2O) was prepared using a Medica® Pro single high flow purification system from ELGA LabWater (Celle, Germany). Reference material of the opioids and designer stimulants included in the study (Table 1 and 2) were obtained from certified reference material retailers or obtained online as 'research chemical'. 'Research chemicals' were evaluated for identification and purity using GC-MS, LC-QTOF-MS, and NMR analysis before use.

Solutions

Working solutions (0.05 ng/mL and 0.5 ng/mL) containing all designer stimulants were prepared in MeOH. The methanolic working solution for opioids contained the opioids at two concentration levels. Level 1 was set to a concentration of 1 µg/mL, while level 2 was set to a concentration of 0.1 µg/mL (Table 2). The internal standard solution (IS) for designer stimulants (concentrations adjusted to yield medium peak intensities with respect to the calibrated concentration range) contained ethylone-D5 (0.1 µg/mL), methamphetamine-D5, methylone-D3, MDE-D5, MDPV-D8, PCP-D5 (0.25 µg/mL each), 2C-C-D6, 2C-I-D6, mephedrone-D3, MDMA-D5 (0.5 µg/mL each), amphetamine-D5, benzylpiperazine-D7, mCPP-D8 (1.0 µg/mL each), norephedrine-D3 (2.5 µg/mL), and methcathinone-D3 (5.0 µg/mL). The IS for opioids contained nortilidine-D3, tapentadol-D3, tramadol-D3, fentanyl-D5, norfentanyl-D5, O-desmethyltramadol-D6, 7-hydroxymitragynine-D3, buprenorphine-D4, dihydrocodeine-D6, EDDP-D3 (perchlorate), hydromorphone-D3, methadone-D9, mitragynine-D3, morphine-D3, norbuprenorphine-D3, oxycodone-D3, oxymorphone-D3, and sufentanil-D5 at 100 ng/mL each. Mobile phase A was deionized water with 1% acetonitrile (ACN), 0.1% formic acid, and 2 mM ammonium formate. Mobile phase B consisted of 0.1% formic acid and 2 mM ammonium formate in ACN.

Instrumentation and methods

The Shimadzu liquid chromatography (LC) system combined a CMB-20AC communications BUS module, a Prominence CTO-20AC column oven (set to 30 °C/40 °C for designer stimulants/opioids), a Nexera X2 SIL-30AC autosampler (10 µl/5 µl injection volume for designer stimulants/opioids, set temperature: 10°C), a DGU-20A5R degasser unit, two Nexera X2 LC-30AD pumps, and a Prominence LC-2AD pump. Coupled to the LC system was a QTRAP® 5500 mass spectrometer (Sciex, Darmstadt, Germany). Ionization was performed in positive electrospray ionization (ESI+), while scheduled multi reaction monitoring (sMRM) was used for data acquisition. MS source parameters were set to the following values:

a) Designer stimulants: Curtain gas 30 psi, ion source gas (1) 40 psi, ion source gas (2) 65 psi, ion spray voltage +4000 V, temperature 550 °C.
b) Opioids: Curtain gas 30 psi, ion source gas (1) 60 psi, ion source gas (2) 70 psi, ion spray voltage +4500 V, temperature 500 °C.

Chromatographic separation for stimulants and opioids was achieved on a biphenyl column (100 x 2.1 mm, 2.6 µm particle size, Phenomenex, Aschaffenburg, Germany) with a corresponding guard column (SecurityGuard™ ULTRA Cartridges UHPLC Biphenyl for 2.1 mm ID columns, Phenomenex, Aschaffenburg, Germany). The chromatographic conditions were as follows:

a) Designer stimulants: The gradient was set to a total flow rate of 0.3 mL/min and started at 5% mobile phase B. After 3 min at 5% mobile phase B was increased to 15% and kept at 15% for 3 min. Within 7 min the percentage of mobile phase B was further increased to 80%. For column cleaning 95% mobile phase B were reached after a total of 18.5 min. After 2.5 min at 95% starting conditions were restored within 0.5 min and kept for additional 4.5 min. For signal enhancement propan-2-ol was added post-column at 0.1 mL/min.
b) Opioids: With a total flow rate of 0.4 mL/min the gradient started at 1% mobile phase B and these conditions were kept for 0.25 min. Within 1.25 min the gradient was increased to 3% mobile phase B. The gradient reached 22.5% mobile phase B after 3 min. Percentage of mobile phase B was further increased to 27.5% within 0.75 min. At the same time the total flow rate was set to 0.45 mL/min. After 8.5 min the gradient reached 32.5% mobile phase B which was further increased to 95% within 1 min. At that time the total flow rate was set to 0.6 mL/min. These conditions were kept for 1.5 min. Finally, starting conditions were restored within 0.1 min and kept for 1.4 min.

Sample preparation

For calibration, 7 calibration samples consisting of 100 µL serum each were spiked with designer stimulants and opioids. The following concentration levels were obtained: 1.0, 5.0, 10, 20, 50, 70, 100 ng/mL (for designer stimulants and opioids level 1) and 10, 50, 100, 200, 500, 700, 1,000 ng/mL (for opioids level 2). The serum was then pipetted on empty filter paper spots (Whatman 903). After drying, 10 µL of both IS solutions were added and the serum spots were transferred in 5 mL Eppendorf tubes. For extraction, 3 mL of MeOH were added and the tubes were ultrasonicated for 15 min. The methanolic extract was transferred into glass vials and subsequently constricted to 100 µL at 40 °C using a gentle stream of nitrogen. After addition of 100 µL of a mixture (*v/v*; 3/1) of propan-2-ol and HCl (≥ 37%) the extract was evaporated to dryness and reconstituted in 100 µL mobile phase A/B (*v/v*; 99/1). For each calibration a blank serum sample (containing only IS) was prepared accordingly. Samples from the subtype C cluster as well as matching non-cluster control samples were spiked with 10 µL of each IS solution and treated as mentioned above.

Evaluation of process efficiency

For evaluation of process efficiency two sets of spiked samples were prepared. Set 1 consisted of 5 blank serum samples (obtained from 5 individuals, 100 µL each) spiked with stimulants and opioids yielding a concentration of 10 ng/mL (100 ng/mL for opioids at level 1). Set 2 consisted of 100 µL MeOH spiked with opioids and designer stimulants and subsequently evaporated to dryness. Process efficiency was evaluated by comparison of absolute peak areas of set 1 and set 2.

Table 1: Included designer stimulants and the mean process efficiency calculated for each analyte.

Designer stimulants	Process efficiency [%]
2,5-DMA	72
2/3/4-Fluoroamphetamine	52
2/3/4-Fluoromethamphetamine	46
2/3/4-Methylmethcathinone	37
2-AI	52
2-FMC	n.e.
2-MAPB	60
2-MeO-Ketamine	56
3,4-CTMP	2.0
3,4-DMA	88
3,4-DMMC	50
3,4-MeO-a-PHP	31
3/4-Me-Buphedrone	49
3-Cl-Methcathinone	19
3-FMC	20
3-F-Phenetrazine	60
3-FPM	61
3-MeO-MC	39
4-APDB	n.e.
4-CAB	61
4-Cl-Methamphetamine	57
4-Cl-Methcathinone	26
4-Cl-PVP	10
4-Ethylethcathinone	56
4-Ethylmethcathinone	45
4-F-a-PBP	33
4-F-a-PVP	33
4-F-BF	40
4-F-Buphedrone	45
4-F-Ethylphenidate	49
4-F-IPV	40
4-FMC	33
4-F-Methylphenidate	73
4-F-PV8	24
4-F-PV9	10
4-Me-Methylphenidate	0
4-Me-N-ethylnorpentedrone	32
4-MeO-BF	39
4-MeO-PV9	28
4-MeO-PVP	43
4-Me-Pentedrone	56
4-Me-Phenmetrazine	65
4-Me-PHP	41
4-MMA	54
4-MTA	815
5-APB	57
5-APDI	63
5-BPDI	36
5-DBFPV	33
5-IAI	77
5-IT	0
5-MAPDB	70
5-MBPB	57
5-PPDI	48
6/5/4-EAPB	64
6/5/4-MAPB	59
6-APB	61
6-APDB	75
7-APDB	66
a-/Naphyrone	29
a-ET	67
AH-792	43
ALEPH-2	415
ALEPH-4	224
a-Me-AHP	47
Amphetamine	54
a-PAVP	47
a-PHP	26
a-PNP	21
a-PVP	48
Benzylpiperazine	27
bk-MDDMA	73
Buphedrone	45

Designer stimulants	Process efficiency [%]
Butylone	70
Cathine	57
Cathinone	43
Cl-Pseudoephedrine	2.0
DB-MDBP	74
DBZP	83
Desoxypipradrol	50
Dimethocaine	0
Dimethylcathinone	40
Diphenidine	50
Ephedrine	63
Etaqualone	51
Ethcathinone	41
Ethylamphetamine	52
Ethylnaphtidate	50
Ethylone	72
Ethylphenidate	47
Isopentedrone	19
Isophenmetrazine	64
Isopropylphendiate	54
Ketamine	72
mCPP	72
MDA	65
MDAI	66
MDAT	91
MDMA	70
MDPBP	71
MDPHP	38
MDPPP	72
MDPV	55
MEAI	77
Mebroqualone	58
Mephtetramine	28
Methamnetamine	40
Methamphetamine	53
Methcathinone	38
Methiopropamine	49
Methoxetamine	73
Methoxphenidine	41
Methylnaphtidate	60
Methylone	65
Methylphenidate	70
MDE	41
NEB-Indane-analog	42
N-Ethylpentylone	75
N-Ethylphenmetrazole	52
Nitracaine	0
N-Me-2AI	54
N-Me-bk-MMDA-2	57
Norephedrine	46
NRG-3	38
PCP	42
Pentedrone	43
Pentylone	70
Phenetrazine	60
PMA	66
PMMA	64
Propylphendiate	59
PV9	24
Pyrovalerone	27
Ritalinic acid	78
TFMPP	66
TMA (3,4,5)	101
TMA-2 (2,4,5)	97
TMA-6 (2,4,6)	89

Table 2: Included opioids, their concentration level, and the mean process efficiency calculated for each analyte.

Opioids	Opioid level	Process efficiency [%]
2-F-Isobutyrfentanyl	2	64
3,4-Methylendioxy-U-47700	1	59
3-Methylfuranylfentanyl	2	68
4-ANPP	2	38
4-Cl-Isobutyrfentanyl	2	72
4-F-Butyrfentanyl	2	66
4-MeO-Butyrfentanyl	2	70
7-Hydroxymitragynine	1	0
Acetylfentanyl	2	51
Acryloylfentanyl	2	53
AH-792	1	66
Alfentanil	2	53
Benzodioxolfentanyl	2	76
Benzylfentanyl	2	54
Buprenorphine	2	0
Butyrfentanyl	2	67
Carfentanil	2	66
Codeine	1	85
Cyclopentylfentanyl	2	79
Cyclopropylfentanyl	2	60
Desomorphine	1	63
Despropionyl-o-F-fentanyl	2	24
Dextrometorphan	1	57
Dihydrocodeine	1	77
Dihydromorphine	1	62
EDDP	1	41
Fentanyl	2	65
Furanylethylfentanyl	2	40
Furanylfentanyl	2	68
Furanylfentanyl 3-CA-isomer	2	58
Hydrocodone	1	49
Hydromorphone	1	46
Loperamide	1	76
MeO-Acetylfentanyl	2	49
Meptazinol	1	78
Methadone	1	74
Mitragynine	1	30
Morphine	1	74
MT-45	1	69
M-U-47700	1	64
N,N-Bidesmethyl-U-47700	1	70
Nalbuphine	1	61
Naloxone	1	39
Naltrexone	1	45
N-Desmethyltapentadol	1	57
N-Desmethyl-U-47700	1	52
Norbuprenorphine	2	0
Norcodeine	1	92
Norfentanyl	2	50
Normorphine	1	64
Noroxycodone	1	36
Nortilidine	1	66
Noscapine	1	62
Ocfentanil	2	0
O-Desmethyltramadol	1	65
Oxycodone	1	47
Oxymorphone	1	44
Papaverine	1	58
Pentazocine	1	57
Pethidine	1	55
Pholcodine	1	102
Remifentanil	2	67
Sufentanil	2	73
Tapentadol	1	63
THF-fentanyl	2	60
Tilidine	1	56
Tramadol	1	69
U-47700	1	66
U-48800	1	57
U-49900	1	68
Valerylfentanyl	2	61
W-18	2	0